International Journal of Pharmaceutics, 99 (1993) 165–172

IJP 03227

Intranasal absorption of angiopeptin: in vitro study of absorption and enzymatic degradation

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(Received 6 January 1993)
(Accepted 17 February 1993)

Key words: Nasal formulation; Peptide; Ussing chamber; Absorption; Absorption enhancer; Enzymatic degradation; Rabbit

Summary

The enzymatic degradation and absorption of angiopeptin (an octapeptide) were studied in vitro. Human nasal wash, rabbit nasal homogenate and supernatant were used as enzyme preparations and the Ussing chamber was used for the absorption studies. No enzymatic degradation was observed as degradation rates of 0.020 μg/h in human nasal wash, 0.046 μg/h in rabbit nasal homogenate and 0.054 μg/h in supernatant, the initial concentration being 0.20 μg/ml, were the same or lower than those observed in control experiments. Degradation is probably caused by hydrolysis, only. The appearance rate (mean $\pm$ S.D.) of angiopeptin from an aqueous solution was 1.82 ± 0.93 μg/h, equivalent to an apparent permeability coefficient of 9.1×10^{-7} cm/s. Addition of 1% glycocholate (GC) enhanced the appearance rate significantly by a factor of 2.7 ($p < 0.05$), whereas no effect was observed after addition of 1 or 5% glycofurol 75 (GF). Short-circuit current and potential difference were measured throughout the penetration study. Addition of 1% GC or 5% GF resulted in significant changes ($p < 0.001$) in electrophysiological properties of the tissue, whereas no changes were observed for 1% GF and control. All in all, the experiments have indicated that angiopeptin is a potential candidate for intranasal application.

Introduction

An increasing number of new drugs are of peptide/protein nature. This change in chemical structure and size of drugs demands development of new delivery systems or use of alternative routes to avoid parenteral administration and still ensure a satisfactory effect. Different routes have been studied and the nasal route is considered to be promising. Using intranasal application it is possible to avoid or reduce some of the disadvantages related to per oral administration of peptides, e.g., enzymatic degradation and first pass metabolism.

Angiopeptin, a synthetic octapeptide (molecular mass 1156 Da), is one of the new promising drugs. The main indication is inhibition of restenose of coronary arteries after angioplasty or heart transplantation. The treatment is expected to take 5–10 days with two to three daily applications of angiopeptin. A nasal formulation for this short treatment period may have advantages over injection, not only due to the reduced pain re-

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lated to nasal application, but especially because nasal application can be performed by an untrained person, and thus the patient may be sent home from hospital much sooner.

The challenge is to make a formulation which is stable and shows a reasonable bioavailability. McMartin et al. (1987) have shown that drugs with a molecular mass greater than about 1000 Da, when administered intranasally, have a very low bioavailability, declining very rapidly with increasing molecular mass. LHRH represents a peptide of the same size as angiopeptin and the bioavailability in humans is only about 1% (Fink et al., 1974). Therefore, it is reasonable to expect the absorption of angiopeptin from a simple solution to be in the same range even though many factors other than size, such as enzymatic degradation and polarity, are of great importance for the bioavailability.

One way to increase bioavailability is by the coadministration of an absorption enhancer, e.g., sodium glycocholate (GC). GC is a well known absorption enhancer (Hirai et al., 1981a; Chan et al., 1988; Pontiroli et al., 1989) with relatively low local toxicological potential (Hirai et al., 1981b; Gizurarson et al., 1990; Hermens et al., 1990), but no data on long-term acceptability are available. Another substance, glycofurol 75 (GF), which is normally used as cosolvent for parenteral solutions of lipophilic drugs, has recently shown promising results in rabbits as an adjuvant (absorption enhancer) in nasal formulations (Bechgaard et al., 1991).

The objective of this study has been to evaluate the possibilities for a nasal formulation of angiopeptin by studying the enzymatic degradation and permeability of angiopeptin in vitro with and without GC and GF.

Materials and Methods

Chemicals

Freeze-dried angiopeptin was kindly provided by Henri Beaufour Institute U.S.A., Inc. The bicarbonate Ringer solution (GR) consisted of HPO_4^{2-} (1.6 mM), $H_2PO_4^-$ (0.4 mM), Mg^{2+} (1.2 mM), Cl^- (122 mM), Ca^{2+} (1.2 mM), K^+ (5 mM), $HCO^-{}_3$ (25 mM), Na^+ (141 mM) and D-(+)-glucose (13 mM), these chemicals except glucose (May & Baker, Dagenham, U.K.) being of analytical grade and obtained from Merck (Darmstadt, Germany). Sigma 7–9® Biochemical buffer, Tris 99.0–99.5%, sodium glycocholate approx. 99% and bovine serum albumin, RIA grade were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Glycofurol 75 was purchased from Roche (Basle, Switzerland). 5 μCi/ml ^{14}C-labelled polyethylene glycol 4000 (PEG-4000) is commercially available from New England Nuclear (Du Pont, Boston, MA, U.S.A.). Phosphoric acid and anhydrous sodium sulphate were of analytical grade and obtained from Merck (Darmstadt, Germany). Acetonitrile of HPLC grade was purchased from Romil Chemicals (Leicester, U.K.). Carbogen (95% O_2/5% CO_2) was obtained from Hede Nielsen A/S (Denmark). Scintillation cocktail (Pico-aqua) was obtained from Packard Instrument BV (Groningen, The Netherlands).

Apparatus

The acrylic Ussing chamber with accessories and scintillation counter were the same as described previously (Bechgaard et al., 1992).

Analysis

The Hitachi HPLC system, used for quantitative analysis of angiopeptin, consisted of a L-6000 pump, a L-4000 variable wavelength UV detector and a Rheodyne® 7125 injection valve (Berkeley, CA, U.S.A.), equipped with a 20 μl loop. The column was a 4 × 250 mm LiChrosorb® RP-18 (5 μm) and the guard column was a LiChroCART® 4–4 from Merck (Darmstadt, Germany).

The mobile phase was a mixture of two solutions A + B (5 + 3). Solution A (pH 2.5) consisted of 0.04 M phosphoric acid, 0.2 M sodium sulphate and 10% acetonitrile. Solution B was a 50% acetonitrile/water solution. Other conditions were: detection at 214 nm; flow rate, 1 ml/min; column temperature, 25°C and injection volume, 20 μl. The retention time was about 8 min. Sample concentrations were calculated on the basis of peak height relative to external angiopeptin standards.

The detection limit was about 0.02 μg/ml and the precision about 10%.

Samples from the mucosal side were diluted 1 + 49 with mobile phase.

Tissue preparation

Rabbit nasal mucosal tissue was obtained from New Zealand White rabbits (about 3 kg) kindly provided by Novo Nordisk A/S (Denmark). Immediately after killing, a longitudinal incision through the lateral wall was made and the nasal cavity was fully opened. The middle and ventral nasal conchae were removed. The tissue was isolated by cutting along the whole septum then carefully loosening the mucosal tissue from the underlying cartilage and bone. The tissue was immediately placed in oxygenated GR or homogenized.

Isolation of nasal enzymes

The procedure described by Stratford and Lee (1986) was used with a few modifications. About 100 mg rabbit nasal mucosal tissue was homogenized for 5 min in 2 ml 0.1 M Tris buffer at 4°C using an Ultra-turax (TP 18/10 no. 71713) homogenizer. 2 ml Tris buffer was added and the mixture was homogenized for 2 min using a manual teflon-glass homogenizer. To obtain the supernatant, the homogenate was centrifuged at 7000 rpm (about $4000 \times g$) in a Sigma refrigerated centrifuge (type 202 MK) at 4°C for 10 min. The supernatant was used directly in the enzymatic degradation study.

Protein content was determined as described by Miller (1959) with slight modification.

Collection of human nasal wash

Samples of human nasal wash were collected by use of a modified nasal washing method introduced by Rossen et al. (1965). While the volunteer was in a Moffats position (Canciani and Mastella, 1988) with the head inclined forward, 2 ml of isotonic sodium chloride solution was instilled into each nasal cavity. After 1 min, specimens consisting of saline and a variable amount of secretion were collected into small glass vials. The specimens obtained were used immediately after sampling. Protein content was determined as mentioned above.

Enzymatic degradation study

The study was conducted in glass vials (Maple Leaf Brand 12 × 75 mm, MacMillan Bathurst Inc., Canada) at 37°C. 1 ml of 0.1 M Tris buffer and 0.5 ml enzyme preparation were mixed and preheated to 37°C. The reaction was initiated by adding 1 ml of 0.5 or 1.25 μg angiopeptin/ml 0.1 M Tris buffer. At different times during 1 h samples (25 μl) were withdrawn for HPLC analysis.

Adsorption study

The study was performed in the Ussing chamber, without tissue, thermostated at 37°C and supplied with carbogen. 2 ml of 0.3 w/v% albumin in GR (GRA) was added to the chamber and after 1 h 200 μl was displaced with a solution containing 5 or 2 μg angiopeptin/ml resulting in a final concentration of 0.5 or 0.2 μg/ml. At different times samples (25 μl) were collected for angiopeptin analysis. The sample volumes were not replaced.

Penetration study

The tissue preparation and Ussing chamber set up were as described previously (Bechgaard et al., 1992). The tissue was preincubated 60 min for stabilisation. GR and GRA were added to the mucosal and serosal side, respectively. In this period and during the penetration study the transepithelial potential difference (PD) and the short-circuit current (I_{sc}) were measured. After preincubation 750 μl GR was replaced with a solution containing 667 μg angiopeptin/ml resulting in a final concentration of 500 μg angiopeptin/ml at the mucosal side. GC and GF were added at both sides after 55 min preincubation in the respective studies. At different times during a 90 min period samples (25 μl) were withdrawn for analysis of angiopeptin. Samples from both the mucosal and serosal side were collected. The sample volumes were replaced.

Because of analytical interference from GC the penetration study with this enhancer was

conducted in a different way (fewer samples and no disappearance study).

Disappearance study

To determine the possible disappearance rate of angiopeptin from the serosal side, 2.5 μg angiopeptin was added to the serosal side immediately after the 90 min absorption study. Samples (25 μl) were withdrawn three times during a 31 min period. After the disappearance study the tissue integrity was assessed with PEG-4000 as described by Bechgaard et al. (1992).

Calculation

The degradation rate (according to the enzymatic degradation study) is equal to $-\alpha \times 60$ min/h, where α is the slope of the regression line.

The concentrations of angiopeptin during the penetration study were corrected for dilution using the following equation:

$$Q = V_s\left(\sum_{n=1}^{n} C_{n-1}\right) + C_n V_t$$

where Q is the total amount of angiopeptin, V_s denotes the sample volume, V_t is the chamber volume and $C_{1,2,\ldots,n}$ is the concentration of sample $1,2,\ldots,n$.

The appearance rate is equal to $\alpha \times 60$ min/h, where α is the slope of the regression line using measured values from 35–85 min (15–92 min for experiments with GC).

The correction for degradation and/or adsorption is calculated from the following equation:

$$\text{Corr}(\%/\text{h}) = (B - C)\frac{100}{\frac{1}{2}(A+B)} \cdot \frac{60}{t_b - t_a}$$

where A and B are the theoretical values at 90 and 121 min, respectively. A is equal to $0.9 \times$ (recovery at 85 min $+ \alpha \times 5) + 5‰$ and $B = A + \alpha \times 31$. C is the measured value at 121 min. t_a and t_b are the times corresponding to A and B, respectively.

The apparent permeability coefficient (P_{app}) was calculated using the following equation:

$$P_{app} = \frac{dQ}{dt} \cdot \frac{1}{CA} \quad (\text{cm/s})$$

where dQ/dt is the slope of the regression line, C represents the concentration at the mucosal side and A is the surface area (0.5 cm^2) of the exposed nasal mucosal tissue.

Results and Discussion

Enzymatic degradation study

As seen from Fig. 1 and Table 1 no enzymatic degradation of angiopeptin was observed. How-

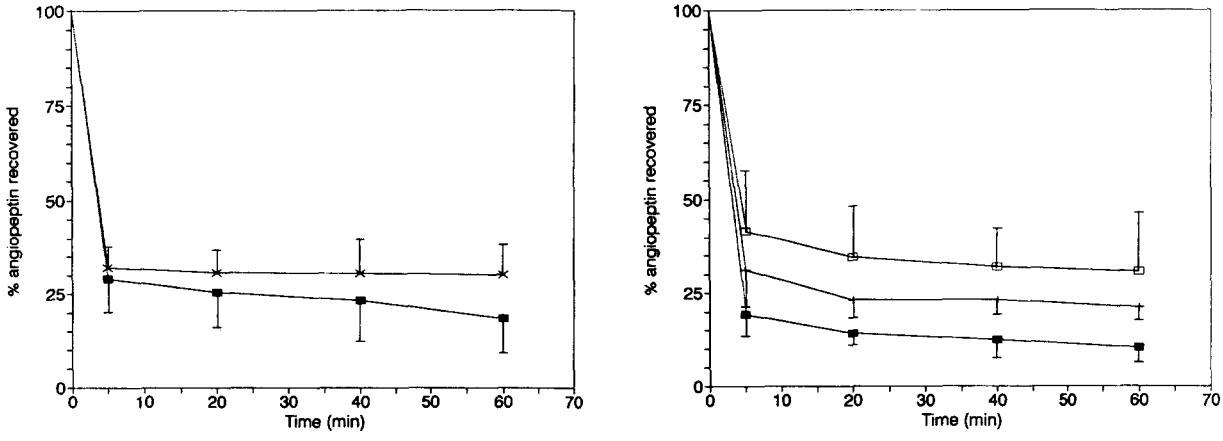

Fig. 1. Degradation (mean ± S.D.) of angiopeptin (0.2 μg/ml) in the presence of human nasal wash (left) (×), rabbit nasal homogenate (right) (+) or rabbit nasal supernatant (right) (□); control (■).

ever, a large initial drop in concentration followed by a much slower linear decrease was observed. The degradation rate (Table 1) is only 0.020 μg/h in human nasal wash, 0.046 μg/h in rabbit nasal homogenate and 0.054 μg/h in the supernatant, the initial concentration being 0.2 μg/ml, and 0.208 μg/h in the homogenate, when the initial concentration is 0.5 μg/ml. The degradation rate in human nasal wash is nevertheless significantly lower ($p < 0.05$) than the control. The observed degradation is expected to be hydrolysis only.

The stability of angiopeptin against enzymatic degradation is not unexpected as the N-terminus is alkylated, the C-terminus is altered by amide formation and some of the amino acids have a D configuration instead of the normal L configuration.

The experiments with enzymes show a smaller initial drop than the control experiments, probably because of a reduction in adsorption to the vials. This could be due to competition for binding sites between angiopeptin and the proteins from the enzyme preparations, as a normal way to prevent adsorption of peptides is by the addition of albumin. In agreement with this, experiments with Tris buffer supplemented with 0.3% albumin show an initial drop of about 10% only (data not shown).

Adsorption study

The adsorption/degradation of angiopeptin in the Ussing chamber, without tissue, is less than 15% per 90 min (initial concentration 0.2 μg/ml) and less than 5% per 90 min at an initial concentration of 0.5 μg/ml. Equal amounts per 90 min (0.025–0.030 μg) disappear in the experiments, and these are in the same range as the degradation rates determined in the enzymatic degradation study.

In contrast to the enzymatic degradation study no initial drop in concentration was observed. This observation is due to the difference in materials (glass vs acrylic) and/or addition of 0.3% albumin in the adsorption study.

Penetration and disappearance study

Addition of 1% GC significantly ($p < 0.05$) enhanced the appearance rate of angiopeptin by a factor of 2.7 (Table 4), whereas addition of 1 or 5% GF did not influence the appearance rate.

The mechanism by which bile salts enhance the penetration of drugs through mucous membranes seems to be due to a combination of effects. Some of the mechanisms of GC are known. It reduces the viscosity and elasticity of mucus (Martin et al., 1978), inhibits proteolytic enzymes (Hirai et al., 1981b) and alters membrane structure (Hirai et al., 1981b). As angiopeptin has been shown to be stable against enzymatic degradation, and the presence of mucus at the isolated nasal tissue is expected to be minimal because of washing during and after isolation, the observed absorption enhancing effect of GC is probably due to alteration of membrane integrity.

TABLE 1

Influence of human nasal wash, rabbit nasal homogenate or supernatant at the degradation rate of angiopeptin

Experiment	*n*	Concentration (μg/ml)	Degradation rate (μg/h) (±S.D.)	Protein content (mg/ml) (±S.D.)	*t*-test [a]
Human nasal wash	6	0.2	0.020 ± 0.017	0.362 ± 0.058	$p < 0.05$
Control	6	0.2	0.054 ± 0.022	–	–
Homogenate	5	0.2	0.046 ± 0.036	3.700 ± 1.592	$p > 0.05$
Supernatant	3	0.2	0.056 ± 0.008	1.572 ± 0.116	$p > 0.05$
Control	6	0.2	0.047 ± 0.022	–	–
Homogenate	2	0.5	0.208 ± 0.184	3.211 ± 1.463	$p > 0.05$
Control	3	0.5	0.132 ± 0.070	–	–

[a] Degradation rate assessed by two-sample *t*-test.

5% GF added to an aqueous solution of human insulin in vivo has been shown to increase the nasal bioavailability in rabbits substantially (Bechgaard et al., 1991). The mechanism involved is not known. In contrast to the promising results with insulin, no effect of GF on the appearance rate of angiopeptin was seen. The different observations with GF can be due to the fact that the mechanism by which GF enhances absorption is only detectable in vivo (e.g., influence on the mucociliary clearance or viscosity of mucus) or is simply due to a difference in the nature of the peptide (e.g., aggregation).

The amount of angiopeptin (mean) penetrating the isolated rabbit nasal tissue is 0.20% during 85 min from an aqueous solution (Table 2). The same value after addition of 1% GF, 5% GF or 1% GC is 0.22%, 0.13% and 0.65% (during 92 min), respectively (Tables 2 and 3).

The corresponding value for insulin (molecular weight 5-times higher) is 0.05% (Bechgaard et al., 1992), i.e., 4-times less. Other experiments with insulin have shown that the amount penetrating nasal tissue is much smaller in vitro than in vivo. As the absorption of LHRH has been shown to be about 1% in vivo (Fink et al., 1974), it would be reasonable to expect the absorption of angiopeptin to be in the same range in vivo or even higher. Therefore, when comparing with LHRH, the possibilities for making a nasal formulation of angiopeptin seem reasonable.

In similar experiments, Hersey and Jackson (1987) determined a P_{app} of 4.3×10^{-6} cm/s for an octapeptide (cholecystokinin), i.e., 5-times higher than for angiopeptin ($P_{app} = 9.1 \times 10^{-7}$ cm/s). One among several possible explanations for the large difference in P_{app} for cholecystokinin and angiopeptin could be that they used isotope fluxes to determine P_{app}. A possible degraded fraction, which penetrates more rapidly, is then included in the P_{app} for cholecystokinin, whereas P_{app} for angiopeptin is determined from measurement of the intact drug only.

TABLE 3

Angiopeptin at serosal side in ‰ of the initial mucosal side concentration (500 μg / ml) at different times (min) during the experiment

Experiment	Serosal side (‰ recovered at min)		
	15	55	92
1% glycocholate	0.06	2.35	5.94
1% glycocholate	0.28	2.25	7.07
1% glycocholate	0.16	3.40	6.89
1% glycocholate	0.08	0.78	1.99
1% glycocholate	0.03	1.07	3.42
1% glycocholate	0.88	7.44	13.55

TABLE 2

Angiopeptin at serosal side in ‰ of the initial mucosal side concentration (500 μg / ml) at different times (min) during the experiment

Experiment	Serosal side (‰ recovered at min)							
	15	35	55	70	85	96	106	121
Control	0.00	0.20	0.77	1.14	1.57	6.70	5.70	7.06
Control	0.04	0.21	0.67	1.07	1.30	5.76	5.86	7.40
Control	0.04	0.30	0.83	1.50	1.78	5.58	4.78	6.84
Control	0.32	0.89	1.55	1.87	2.41	7.08	6.04	7.42
Control	0.45	1.42	2.49	3.45	4.33	9.00	8.54	9.52
Control	0.00	0.07	0.24	0.49	0.69	5.30	4.70	5.52
1% glycofurol	0.14	0.50	1.10	1.44	1.88	7.64	7.58	8.08
1% glycofurol	0.04	0.21	0.43	0.60	0.78	4.76	4.70	4.42
1% glycofurol	0.14	1.02	1.77	2.67	3.80	7.62	8.14	9.14
5% glycofurol	0.00	0.06	0.17	0.27	0.40	5.97	2.52	3.82
5% glycofurol	0.00	0.26	0.59	0.81	1.32	5.50	5.02	5.20
5% glycofurol	0.16	0.60	1.20	1.59	2.15	6.58	6.64	7.22

At 90 min 5‰ angiopeptin was added to the serosal side to estimate the correction for degradation.

TABLE 4

Influence of GC and GF at appearance rate, corrected appearance rate and apparent permeability coefficient for angiopeptin

Experiment	Appearance rate (μg/h)	Corrected appearance rate (μg/h)	P_{app} ($\times 10^7$) (cm/s)	t-test [a]
Control	1.82 ± 0.93	2.18 ± 1.26	9.12 ± 5.15	–
1% GC	4.84 ± 2.89	–	26.90 ± 16.08	$p < 0.05$
1% GF	1.88 ± 1.34	2.28 ± 1.65	9.62 ± 6.76	$p > 0.05$
5% GF	1.15 ± 0.72	1.56 ± 0.81	5.78 ± 3.77	$p > 0.05$

[a] Appearance rate assessed by two-sample t-test.

The experiments were conducted in the Ussing chamber mounted with rabbit nasal tissue. All values are expressed as mean ± S.D. (GC, sodium glycocholate; GF, glycofurol 75).

In an attempt to correct for possible degradation and/or adsorption at the serosal side, 2.5 μg angiopeptin/ml (equivalent to 0.5% of the initial mucosal concentration) was added immediately after the 90 min penetration study. The corrected appearance rates (Table 4) are 2.18, 2.28 and 1.56 μg/h for a solution without enhancer, with 1% GF or with 5% GF, respectively (i.e., an increase of 20–36%). This insignificant correction indicates that the experiment is relatively well controlled with respect to degradation and adsorption, even at low concentrations.

As seen from Tables 2 and 3 the amount of angiopeptin penetrating the isolated nasal tissue varies considerably. This large variation is partly caused by variation in thickness of the isolated tissue, and may eventually be corrected for by coadministration of a standard absorbable reference. The variation makes it time consuming to use the in vitro system to screen for absorption enhancers as it demands many experiments (probably $\geqq 6$) to show any statistical significance. Other experiments have shown that it is difficult to identify some absorption enhancers in the in vitro system (Bechgaard et al., 1993).

Table 5 shows the electrophysiological data of the isolated rabbit nasal tissue before and after the penetration study. There is no significant difference ($p > 0.05$) in the initial I_{sc} and PD for the four different experiments or in I_{sc} and PD before and after the penetration study in control experiments and experiments with 1% GF. Addition of 1% GC or 5% GF to the solution resulted in a rapid decline in I_{sc} and PD (both reached nearly zero in less than 15 min). This drop indicates a local effect on the cells, however, it is not yet possible to predict whether this has any consequence in vivo, where the formulation is diluted due to mucosal secretion and the tissue repair mechanisms are normal. Wheatley et al. (1988) have shown a similar but weaker effect of 1% GC on ovine nasal mucosa. Gizurarson et al. (1990)

TABLE 5

Short-circuit current ($\mu A/cm^2$) and potential difference (mV) across rabbit nasal tissue mounted in the Ussing chamber

Experiment	After preincubation		After penetration study	
	$\mu A/cm^2$	mV	$\mu A/cm^2$	mV
Control	63.3 ± 27.0	3.6 ± 2.4	70.3 ± 30.3	4.2 ± 2.9
1% glycocholate	84.0 ± 66.0	4.6 ± 2.2	0.3 ± 0.8	0.0 ± 0.0
1% glycofurol	104.0 ± 29.1	5.4 ± 3.9	82.0 ± 20.9	3.9 ± 1.8
5% glycofurol	101.3 ± 56.6	7.9 ± 6.0	2.0 ± 2.0	0.2 ± 0.2

All values are expressed as mean ± S.D.

have found that 1% GC does not influence the mucociliary transport rate, measured on a frog palate model.

Conclusion

The results indicate that angiopeptin is a potential candidate for intranasal administration. Angiopeptin has been shown to be quite stable against enzymatic degradation; addition of an enzyme inhibitor is unnecessary. It has been shown to be possible to increase the appearance rate by coadministration of 1% GC whereas GF (1 or 5%) has no influence on the appearance rate. Unfortunately, 1% GC has also been shown to change the electrophysiological properties of the nasal tissue in vitro, but the clinical relevance with respect to local toxicity is not yet known.

References

Bechgaard, E., Gizurarson, S. and Hjortkær, R.K., A pharmaceutical preparation. *Danish Patent no. PCT/DK 91/00119,* 1991, pp. 1–69.

Bechgaard, E., Gizurarson, S., Jørgensen, L. and Larsen, R., The viability of isolated rabbit nasal mucosa in the Ussing Chamber and the permeability of insulin across the membrane. *Int. J. Pharm.,* 87 (1992) 125–132.

Bechgaard, E., Jørgensen, L., Larsen, R., Gizurarson, S., Carstensen, J. and Hvass, A., Insulin and didecanoyl-L-α-phosphatidylcholine: in vitro study of the transport through nasal mucosal tissue from rabbits. *Int. J. Pharm.,* 89 (1993) 147–153.

Canciani, M. and Mastella, G., Efficacy of beclomethasone nasal drops, administered in the Moffat's position for nasal polyposis. *Acta Pediatr. Scand.,* 77 (1988) 612–613.

Chan, R.L., Henzl, M.R., LePage, M.E., LaFargue, J., Nerenberg, C.A., Shabbir, A. and Chaplin, M.D., Absorption and metabolism of nafarelin, a potent agonist of gonadotropin-releasing hormone. *Clin. Pharmacol. Ther.,* 44 (1988) 275–282.

Fink, G., Gennser, G., Liedholm, P., Thorell, J. and Mulder, J., Comparison of plasma levels of luteinizing hormone-releasing hormone in men after intravenous or intranasal administration. *J. Endocr.,* 63 (1974) 351–360.

Gizurarson, S., Marriott, C., Martin, G.P. and Bechgaard, E., The influence of insulin and some excipients used in nasal insulin preparations on mucociliary clearance. *Int. J. Pharm.,* 65 (1990) 243–247.

Hermens, W.A.J.J., Hooymans, P.M., Verhoef, J.C. and Merkus, F.W.H.M., Effects of absorption enhancers on human nasal tissue ciliary movement in vitro. *Pharm. Res.,* 7 (1990) 144–146.

Hirai, S., Yashiki, T. and Mima, H., Effect of surfactants on the nasal absorption of insulin in rats. *Int. J. Pharm.,* 9 (1981a) 165–172.

Hirai, S., Yashiki, T. and Mima, H., Mechanisms for the enhancement of the nasal absorption of insulin by surfactants. *Int. J. Pharm.,* 9 (1981b) 173–184.

Hersey, S.J. and Jackson, R.T., Effect of bile salts on nasal permeability in vitro. *J. Pharm. Sci.,* 76 (1987) 876–879.

Martin, G.P., Marriott, C. and Kellaway, I.W., Direct effect of bile acids and phospholipids on the physical properties of mucus. *Gut,* 19 (1978) 103–107.

McMartin, C., Hutchinson, L.E.F., Hyde, R. and Peters, G.E., Analysis of structural requirements for the absorption of drugs and macromolecules. *J. Pharm. Sci.,* 76 (1987) 535–540.

Miller, G.L., Protein determination for large numbers of samples. *Anal. Chem.,* 31 (1959) 964.

Pontiroli, A.E., Alberetto, M., Calderara, A., Pajetta, E. and Pozza, G., Nasal administration of glucagon and human calcitonin to healthy subjects: a comparison of powders and spray solutions and of different enhancing agents. *Eur. J. Clin. Pharmacol.,* 37 (1989) 427–430.

Rossen, R.D., Butler, W.T., Cate, C.F., Szwed, C.F. and Counh, R.B., Protein composition of nasal secretion during respiratory virus infection. *Proc. Soc. Exp. Biol. Med.,* 119 (1965) 1169–1176.

Stratford, R.E. and Lee, V.H.L., Aminopeptidase activity in homogenates of various absorptive mucosae in the albino rabbit: implications in peptide delivery. *Int. J. Pharm.,* 30 (1986) 73–82.

Wheatley, M.A., Dent, J., Wheeldon, E.B. and Smith, P.L., Nasal drug delivery: An in vitro characterization of transepithelial electrical properties and fluxes in the presence or absence of enhancers. *J. Controlled Release,* 8 (1988) 167–177.